Cell

Fatty Acid Synthesis by Elongases in Trypanosomes

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DOI 10.1016/j.cell.2006.06.045

SUMMARY

All eukaryotic and prokaryotic organisms are thought to synthesize fatty acids using a type I or type II synthase. In addition, eukaryotes extend pre-existing long chain fatty acids using microsomal elongases (ELOs). We have found that *Trypanosoma brucei*, a eukaryotic human parasite that causes sleeping sickness, uses three elongases instead of type I or type II synthases for the synthesis of nearly all its fatty acids. Trypanosomes encounter diverse environments during their life cycle with different fatty acid requirements. The tsetse vector form requires synthesis of stearate (C18), whereas the bloodstream form needs myristate (C14). We find that trypanosome fatty acid synthesis is modular, with ELO1 converting C4 to C10, ELO2 extending C10 to C14, and ELO3 elongating C14 to C18. In blood, ELO3 downregulation favors myristate synthesis, whereas low concentrations of exogenous fatty acids in cultured parasites cause upregulation of the entire pathway, allowing the parasite to adapt to different environments.

INTRODUCTION

Trypanosoma brucei, the sleeping sickness parasite, divides its life cycle between the tsetse fly vector and the mammalian host. The bloodstream form (BSF) evades the host's immune response by switching surface coats composed of 10^7 variant surface glycoprotein (VSG) molecules (Donelson, 2003). Each VSG is tethered to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor whose fatty acids (FAs) are exclusively myristate (tetradecanoate), a 14-carbon saturated FA (Ferguson and Cross, 1984). Myristate, via myristoyl-CoA, is incorporated into the GPI precursor in a microsomal FA remodeling reaction that replaces other long chain FAs with myristate (Masterson et al., 1990). In contrast, tsetse fly procyclic forms (PCFs) do not myristoylate the GPI anchors of their surface glycoproteins (Field et al., 1991).

BSF trypanosomes need massive quantities of myristate for their GPI anchors. It was long believed that they were unable to synthesize FAs de novo (Dixon et al., 1971) and that they acquired all FAs, including myristate, from the host blood. Although trypanosomes salvage myristate efficiently (Doering et al., 1993), the myristate concentration in blood is hardly adequate to support GPI myristoylation at high parasite densities (Paul et al., 2001). Several years ago we questioned whether trypanosomes synthesize FAs and discovered that indeed they do (Morita et al., 2000). We developed a cell-free system for FA synthesis containing trypanosome membranes (a source of enzymes), butyryl-CoA (the four-carbon primer; acetyl-CoA does not prime), malonyl-CoA (the two-carbon donor), and NADPH. The end product with BSF membranes is predominantly myristate, which can be used for GPI anchors. In contrast, the longest end product in PCFs is stearate (octadecanoate; C18).

In other cell types, FAs are made by a type I or type II FA synthase (Smith, 1994; White et al., 2005). Type I enzymes, found in the cytosol of mammals and fungi, have multiple catalytic activities residing on separate domains of one or two large polypeptides (Smith, 1994). An exception is found in *Musca domestica* and *Blattella germanica*. In addition to a cytosolic type I FA synthase, both insects utilize a type I FA synthase that is a microsomal peripheral membrane protein in the integument to produce methyl-branched FAs (Juarez et al., 1992; Gu et al., 1997). Type II FA synthases, also soluble and found in bacteria, plants, and in eukaryotic organelles of prokaryotic origin, have similar catalytic activities, but each is on a separate polypeptide (White et al., 2005; Zhang et al., 2005). In both systems the growing chain is esterified to acyl-carrier protein (ACP) or an ACP-like domain.

So which system makes FAs in *T. brucei*? The *T. brucei* genome does not encode a type I FA synthase, but does predict a type II system (Paul et al., 2001). Thus, we naturally assumed that the synthase was type II. However, several observations taken together suggested the type II pathway is not responsible for trypanosome bulk FA synthesis. First, typical type II systems are composed of soluble proteins, yet the trypanosome activity is membrane-associated. Second, type II enzymes and ACP localize to the trypanosome's single mitochondrion (J.S., unpublished data), but the myristate product is needed in the

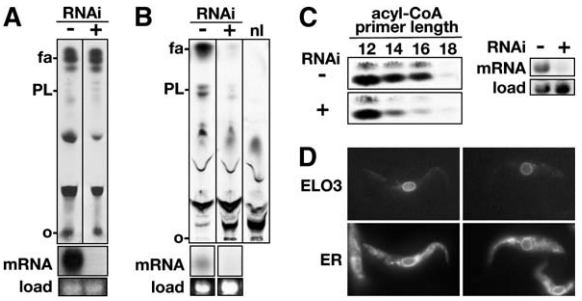

Figure 1. Effect of RNAi on Fatty Acid Synthesis and ELO3 Localization

(A) Upper panel shows effect of ACP RNAi for 2 days on cell-free FA synthesis from butyryl-CoA in PCF trypanosomes (1.3×10^8 cell equivalents/lane). RNAi (+), uninduced control (−). Middle panel is Northern blot showing effect of RNAi on ACP mRNA (~0.9 kb). Load control (load) is ethidium-stained rRNA. fa, free fatty acid products; PL, phospholipids; o, origin.

(B) Same as (A) except ELO1 RNAi was for 4 days (1.2×10^7 cell equivalents/lane). No lysate (nl) reaction does not contain cell membranes. ELO1 mRNA is ~2.0 kb.

(C) Same as (A) except ELO3 RNAi was for 2 days. Left panel shows free FA products (3.5×10^7 cell equivalents/lane) using membranes from uninduced (−) and RNAi-induced (+) cells. Right panel is Northern blot showing effect of RNAi on ELO3 mRNA (~2.1 kb) and load control.

(D) Upper panels show fluorescence of live PCF trypanosomes expressing a C-terminal GFP-tagged ELO3. Lower panels show fluorescence of ER Tracker in the same cells. ELO3-GFP and ER Tracker colocalize to the perinuclear ring (color merge not shown). All cells were stained with ER Tracker, while ELO3-GFP expression was detected only in a small fraction of cells.

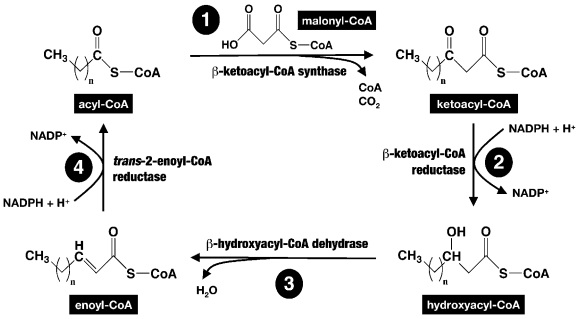

Figure 2. Fatty Acid Elongation Enzyme Reactions

In step 1, β-ketoacyl-CoA synthase (elongase) condenses a long-chain acyl-CoA (where the number 'n' is an even number) with two carbons from malonyl-CoA to form β-ketoacyl-CoA. In step 2, β-ketoacyl-CoA reductase reduces β-ketoacyl-CoA to β-hydroxyacyl-CoA, which in turn is dehydrated in step 3 by β-hydroxyacyl-CoA dehydrase. The resulting enoyl-CoA is reduced by *trans*-2-enoyl-CoA reductase in step 4 to produce a saturated acyl-CoA chain. NADPH is required for steps 2 and 4.

ER where GPI remodeling occurs. Third, triclosan, a type II enoyl-ACP reductase inhibitor, affects cell-free FA synthesis only at high concentrations, suggesting nonspecific inhibition (Paul et al., 2004). Fourth, cerulenin, which inhibits the β-ketoacyl-ACP synthase (KAS; both type I and type II), does not affect cell-free synthesis of intermediates up to C10, although it does inhibit extension beyond C10 (Morita et al., 2000). This specificity of cerulenin could be explained by the existence of two KAS activities; however, the genome predicts only one. Finally, and most compellingly, RNA interference (RNAi) silencing of ACP, a key component of type II systems, has no effect on FA synthesis in the cell-free system (Figure 1A). For these and other reasons we concluded that the type II pathway is mitochondrial and that its products are produced at low levels. Therefore, bulk FA synthesis in trypanosomes must be unconventional, involving neither a type I nor a type II FA synthase. As an alternative we considered the possibility that a microsomal FA elongase system is involved.

Elongase systems in all known cell types extend the products of type I FA synthase (myristate in yeast and palmitate [hexadecanoate; C16] in mammals) to longer-chain FAs (Moon et al., 2001). *Saccharomyces cerevisiae* ELO1–3 extend C14 to C16, C16 to C24, and C24 to C26, respectively (Oh et al., 1997; Toke and Martin, 1996). The chemistry of the elongase reactions (diagrammed in Figure 2) resembles that of type I and type II systems, except the growing acyl chain is esterified to CoA instead of ACP. The ELOs condense malonyl-CoA with the growing acyl chain, extending it by two carbon atoms (Figure 2, step 1). The resulting β-ketoacyl-CoA is then reduced (β-ketoacyl-CoA reductase, step 2), dehydrated (β-hydroxyacyl-CoA dehydrase, step 3), and reduced again (*trans*-2-enoyl-CoA reductase, step 4), producing a longer, saturated acyl-CoA (Leonard et al., 2004). The two reduction steps require NADPH. We now describe the trypanosome's unusual biochemical machinery involving FA elongases for bulk FA synthesis.

RESULTS

Elongases Are Required for Bulk Fatty Acid Synthesis

T. brucei has four candidate *ELO* genes. *ELO1–3* are in tandem on chromosome seven (NCBI AAX70671, AAX70672, and AAX70673) while *ELO4* is on chromosome five (with allelic variants AAX69821 and AAX70768). Like *S. cerevisiae* ELOs, the trypanosome ELOs have multiple predicted transmembrane domains and an HXXHH amino acid motif (Oh et al., 1997). We used RNAi to silence ELO1 in PCF trypanosomes and then assayed membrane isolates for FA synthesis. In contrast to the effect of ACP RNAi, ELO1 knockdown drastically reduced FA synthesis (Figure 1B). RNAi also provided preliminary evidence that other ELOs act on longer acyl chains (Figure 1C). These studies indicated that the ELO pathway is involved in cell-free FA synthesis. To confirm the predicted intracellular localization of one of the enzymes in this pathway, we expressed ELO3 tagged with a C-terminal GFP; fluorescence microscopy showed that it colocalized with ER Tracker predominantly in the perinuclear region

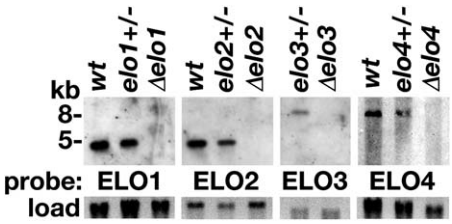

Figure 3. Southern Analysis of BSF *ELO1–4* Genomic Knockouts (*Δelo*)

Genomic DNA from wild-type (*wt*), heterozygous (+/−), and homozygous knockout clones (*Δelo*) was restriction digested with MluI/NotI/XhoI, NdeI/SacI/XhoI, KpnI/PacI/SacI, and MluI/NotI/XhoI, respectively, for ELO1–4 blots. Fragments were detected with appropriate probes. ACP 5′ UTR sequence was used to control for loading (load). Wild-type sample for ELO3 was lost.

(Figure 1D), similar to the localization of the yeast ELO pathway (Kohlwein et al., 2001).

Sequential Fatty Acid Synthesis by ELO1–3

To evaluate more rigorously the role of each ELO in FA synthesis, we made knockout strains for each of the four *ELO* genes (*Δelo*) in BSF trypanosomes. Figure 3 shows Southern blots confirming the knockout of each gene. We assayed membranes from wild-type and knockout strains in the cell-free FA synthesis system (Morita et al., 2000) using acyl-CoA primers of varying chain length. We detected and quantitated the products by thin layer chromatography (TLC) and phosphorimaging (Figure 4A), and we determined the product chain lengths by reverse-phase TLC of their methyl esters (Figure 5A). These data (presented below) show that ELO1 mainly extends C4 to C10, ELO2 extends C10 to C14, and ELO3 extends C14 to C18. Furthermore, the ELO specificities are overlapping. For example, ELO2, which prefers C10- and C12-CoA primers, also has low activity with C8- and C14-CoA.

Using wild-type membranes, we found robust synthesis with primer lengths from four to 12 carbons, though utilization of C4- and C12-CoA primers was about half as efficient as that of the intermediate primers (Figure 4A). Wild-type membranes showed relatively low extension of C14- and C16-CoA, as expected, because BSFs mainly synthesize C14 (Morita et al., 2000). Chain-length analysis of the FA products from these wild-type reactions revealed that C4-CoA is elongated efficiently, mostly to C14, with little accumulation of intermediates (Figure 5A). In contrast, elongation of C6- and C8-CoA appeared less processive, with more accumulation of intermediates. Argentation TLC, a procedure that fractionates FAs by degree of unsaturation, revealed small amounts of unsaturated FAs (Figure 5B); these are likely due to the activity of desaturases on the products of the ELO pathway.

In *Δelo1* membranes, synthesis from primers ranging from C4- to C12-CoA was almost completely lost, whereas synthesis from C14- and C16-CoA was less affected (Figure 4A). Despite these observations, other evidence indicates that ELO1 is mainly involved in synthesis up to

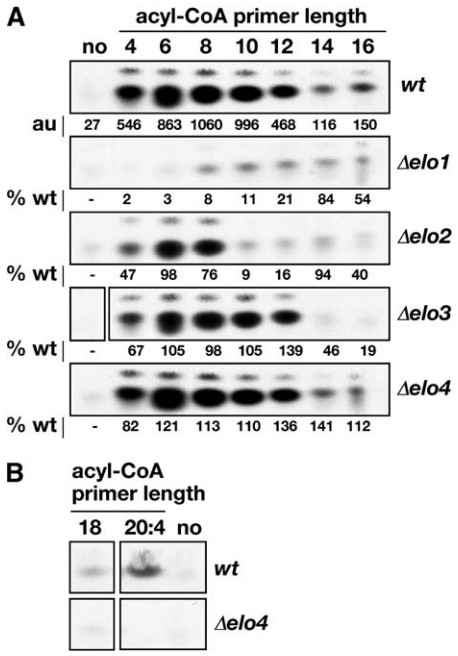

Figure 4. Fatty Acid Synthesis by BSF *Δelo1–4* Membranes from Various acyl-CoA Primers

(A) The segment of the TLC plate with free FAs is shown (1.8×10^7 cell equivalents/lane). The minor upper component is neutral lipid (e.g., triglyceride). Protein per lane ranged from 7.5 to 10.3 μg. The no primer control for *Δelo3* is from a duplicate experiment. Phosphorimager quantitations of FAs are indicated in arbitrary units (au) for wild-type and as a percentage of wild-type for the knockouts (% wt).

(B) Similar to (A) except 1.3×10^7 cell equivalents were loaded per lane and protein was 22 and 15 μg/lane for wild-type and *Δelo4*, respectively. C18-CoA primer was included to control for *Δelo4* membrane activity; the low level of product corresponds to C20 (S.H.L., unpublished data), which is probably due to overlapping ELO3 activity.

C10. As shown in Figures 4A and 5A, BSF *Δelo2* membranes efficiently utilize primers up to C8 (forming C10), an activity that must be due to ELO1. Furthermore, cerulenin does not affect synthesis in the cell-free system up to C10, but blocks further elongation (Morita et al., 2000), suggesting that it inhibits ELO2, but not ELO1. We do not know the reason why BSF *Δelo1* is defective in ELO2 activity; perhaps ELO1 protein is required for downstream ELO activity in vitro.

We used the same approach to study the specificities of ELO2–4. Membranes from *Δelo2* were mainly deficient in extending C10- and C12-CoA primers (Figure 4A). As mentioned, chain-length analysis of these products showed that *Δelo2* stops FA synthesis at C10 with shorter primers (Figure 5A). The minor chain elongation of C10- and C12-CoA in *Δelo2* membranes is likely due to overlapping ELO1 and ELO3 activities. Similarly, *Δelo3* membranes appeared deficient in extending C14- and C16-CoA and chain elongation of shorter primers stopped at C14 (Figures 4A and 5A). The chain-length specificity of ELO3 was confirmed using PCF ELO3 RNAi membranes (Figure 1C). Residual elongation of C14-CoA in

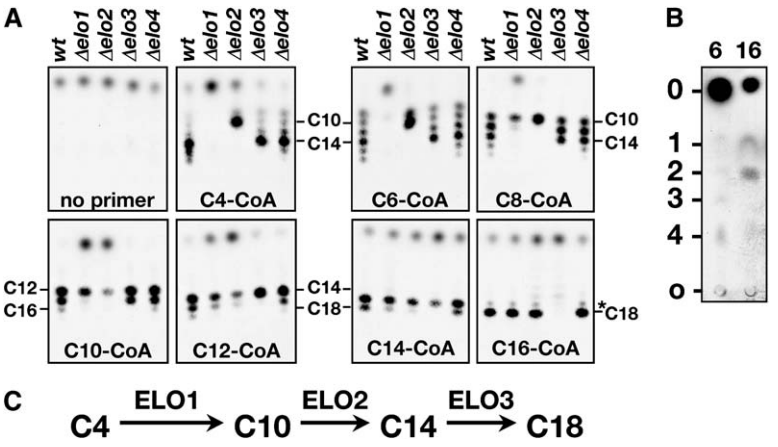

Figure 5. Analysis of Chain Length and Unsaturation of Fatty Acid Synthesis Products

(A) FA methyl esters (~5000 DPM/lane when available) were fractionated on reverse-phase TLC. Thus, intensities are not proportional to products formed. The uppermost band is an aqueous phase contaminant that increases with volume of hexane phase loaded. The band labeled with (*) in the C16-CoA panel is C18:1. We have not characterized the faint ladder of C8 to C18 products observed in *Δelo2* and *Δelo3* lanes (C16-CoA primer) and that is sometimes observed in no primer samples.

(B) Argentation TLC of methyl esters of products from wild-type C6-CoA (6) and C16-CoA (16) primed FA synthesis reactions. Origin, o; saturated species, 0; mono-unsaturated, 1; di-unsaturated, 2; tri-unsaturated, 3; and tetra-unsaturated species, 4.

(C) Substrate preferences for ELO1–3.

Δelo3 membranes is likely due to overlapping ELO2 activity. Substrate preferences for ELO1–3 are summarized in Figure 5C. Taken together, these data indicate that ELO1–3 account for virtually all of the FA synthesis observed in the cell-free system.

ELO4 Elongates Arachidonoyl-CoA

Using primers up to C16-CoA, the products of *Δelo4* membranes resembled those of wild-type, indicating ELO4 is not involved in FA synthesis up to C18 (Figures 4A and 5A). However, ELO4 elongates the unsaturated long chain FA arachidonate (C20:4 or 5,8,11,14-eicosatetraenoate). Although wild-type membranes efficiently elongated arachidonoyl-CoA (Figure 4B) and chain-length analysis revealed a single radiolabeled product that coeluted with C22:4 (S.H.L., unpublished data), *Δelo4* membranes were inactive in this reaction.

Fatty Acid Synthesis in Cultured Parasites

We next tested whether the ELO pathway is responsible for FA synthesis in cultured *T. brucei*. We identified a single gene for enoyl-CoA reductase (*EnCR*; NCBI AAX80213) on chromosome three by homology to that from *S. cerevisiae*, where it functions downstream of all three yeast ELOs (Kohlwein et al., 2001). We found EnCR RNAi cells defective for growth in both low-lipid (Figure 6A) and, to a lesser extent, normal media (S.H.L., unpublished data), indicating that the ELO pathway is essential in PCF trypanosomes. The growth defect was reversed by stearate addition (Figure 6A). This rescue, signifying that inadequate stearate is produced in the RNAi cells, demonstrates that the ELO pathway is involved in stearate synthesis in cultured *T. brucei*. We then assayed RNAi membranes for cell-free FA synthesis and found 77%, 63%, and 54% reduced activity, compared to uninduced control, using C4-, C10-, and C14-CoA primers, respectively (Figure 6B). As with many other trypanosome genes, *EnCR* RNAi was incomplete. Based on the RNAi phenotype, it is likely, as in yeast, that ELO1–3 share a single EnCR. Finally, we measured the effect of EnCR RNAi on FA synthesis in cultured parasites. Since trypanosomes do not take up [^{14}C]acetate (Morita et al., 2000), an FA precursor, we radiolabeled them with [^{14}C]threonine, which is catabolized to glycine and acetyl-CoA (van Weelden et al., 2005). The latter compound is incorporated into FAs following conversion to malonyl-CoA (Klein and Linstead, 1976; van Weelden et al., 2005). We detected all FA synthesis intermediates between C8 and C18 (C6 methyl ester is lost during the hexane extraction), which indicates that the whole pathway is active in the parasite (Figure 6C). Because [^{14}C]threonine labeling of FAs from C8 to C18 is reduced by 48% in normal and 68% in low-lipid medium by EnCR RNAi (Figure 6C, lanes 1 and 2 or 3 and 4), and because RNAi was likely incomplete, the ELO pathway must be responsible at least for the majority of FA synthesis in cultured *T. brucei*.

Regulation of the ELO Pathway

It appears that the *T. brucei* ELO pathway is regulated. Comparison of lanes 1 and 3 in Figure 6C (both uninduced for RNAi) shows that PCF FA synthesis is increased 98% following growth in low-lipid medium, indicating that the entire ELO pathway is upregulated in a low-lipid environment. In a control experiment, we found that [^{14}C]threonine was taken up at a 38% lower rate (average of three time points up to 4.5 hr) by cells grown in low-lipid medium than by those grown in normal medium. Therefore, the higher rate of incorporation of ^{14}C into FAs in low-lipid medium is not due to higher uptake of the [^{14}C]threonine precursor.

Fatty Acid Synthesis in Other Trypanosomatids

The genomes of related disease-causing trypanosomatids *Leishmania major* and *Trypanosoma cruzi* also encode ELO systems. Indeed, like *T. brucei*, their membranes efficiently synthesize FAs from butyryl-CoA but not acetyl-CoA (Figure 7). Although in vitro synthesis activity was more robust in *T. cruzi* than in *L. major*, FA synthesis

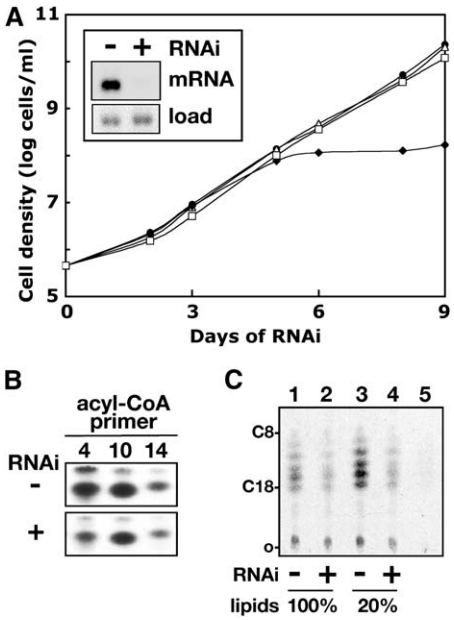

Figure 6. Effect of RNAi Knockdown of Enoyl-CoA Reductase in PCFs

(A) Growth of cells in low-lipid medium in the presence or absence of 35 μM stearate. Uninduced control, no stearate (closed circles); RNAi, no stearate (closed diamonds); uninduced control plus stearate (open triangles); RNAi plus stearate (open squares). Inset is Northern blot showing reduced enoyl-CoA Reductase (EnCR) mRNA (~3.1 kb) after 6 days of RNAi. Load control (load) is ethidium-stained rRNA.

(B) Portion of TLC showing free FA products formed in 10 min with C4, C10, and C14-CoA in uninduced (–) and 5 day RNAi induced (+) membranes (1.6×10^7 cells/lane). Protein per lane was 15.7 μg and 23.1 μg, respectively.

(C) Chain-length analysis of FAs labeled with [^{14}C]threonine after 5 (+) or no (–) days of RNAi (1.5×10^7 cells/lane). Cells were cultured either in normal (100%) or low-lipid (20%) medium. Lane 5 is a zero time point labeling control.

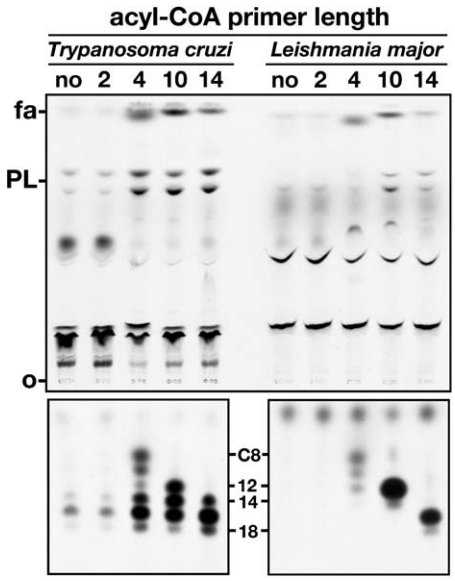

Figure 7. Cell-Free Fatty Acid Synthesis in *T. cruzi* Epimastigotes and *L. major* Promastigotes

FA synthesis reactions using acyl-CoA primers ranging from C2 to C14 were conducted essentially as with *T. brucei* (see Experimental Procedures). Activity in the no primer lane (no) is likely due to endogenous primers. Upper panel is a TLC analysis, while lower panels show chain lengths of products. fa, free fatty acid products; PL, phospholipids; o, origin. Unsaturated FA products such as C18:1 and C18:2 coelute with C16:0 and C14:0, respectively. For all panels, 1.3×10^7 cell equivalents/lane were loaded.

products ranging from C8 to C18 were observed in both systems.

DISCUSSION

A New and Versatile Pathway for Fatty Acid Synthesis

African trypanosomes use an unprecedented mechanism to synthesize FAs. All other cell types, whether prokaryotic or eukaryotic, utilize conventional type I or type II FA synthases. *T. brucei* is the first example of a cell that has adapted microsomal elongases for bulk FA synthesis. We have also presented evidence that two related parasites, *L. major* and *T. cruzi*, utilize elongases for FA synthesis. Whereas the *T. brucei* genome encodes four *ELOs*, *L. major* and *T. cruzi* genomes encode 13 and five, respectively. As with *T. brucei*, the genomes of these related parasites also encode a type II FA synthase, but not a type I. We have found in *T. brucei* that components of the type II system localize to the mitochondrial matrix (K.P. and J.S., unpublished data), and the same is likely true for *L. major* and *T. cruzi*. We also have found that FA products of the trypanosome mitochondrial system are produced at a very low level (J.S., unpublished data). Therefore, nearly all of the FAs synthesized in *T. brucei* are produced by the elongase pathway.

Why do these parasites utilize the elongase pathway for FA synthesis? One obvious advantage is that synthesis is near the site of product utilization. Phospholipid synthesis in the ER membrane can utilize FAs made nearby. Also, GPI myristoylation, which occurs in the ER of BSF trypanosomes, can employ myristate, a major product of the ELO pathway. Another advantage is that the stepwise synthesis of FAs to C10, C14, and finally to C18 allows convenient switching of the products of the pathway. The obvious example is in BSF *T. brucei* where selective downregulation of ELO3 (see below) results in synthesis of myristate. We speculate that some of the 13 ELOs in *L. major* are specialized for synthesis of the long-chain fatty acyl groups and synthesis of the carbon chains of the alkyl groups needed for both glycoinositolphospholipids and the GPI anchors of promastigote surface protease and lipophosphoglycan (Ferguson, 1997).

Regulation of the ELO Pathway

The versatility of the ELO pathway is enhanced by the fact that it can be regulated to suit the parasite's needs. We

found in PCFs that the entire pathway is upregulated when the cells are cultured in low-lipid medium (Figure 6C). There are also previously published observations that can now be explained in terms of regulation of the ELO pathway. These include the facts that the rate of FA synthesis in PCFs, as measured in the cell-free system, is 5.3-fold higher than in BSFs, indicating upregulation of the pathway in PCFs (Morita et al., 2000). Also, the different FA products produced in BSFs and PCFs can be explained by selective up- or downregulation of ELO3 (Morita et al., 2000). Another example comes from our previous report that BSFs grown in medium containing 5% serum efficiently elongate exogenous [^{3}H]myristate to palmitate and stearate, whereas trypanosomes in whole blood do not (Doering et al., 1993). Whole blood has 20 times more palmitate and stearate than media with 5% serum. Therefore, BSF ELO3 must be downregulated during culture in whole blood and upregulated during growth in FA-depleted medium. These findings indicate that trypanosomes regulate FA synthesis by the ELO pathway in response to environmental FAs. We do not yet know the molecular mechanisms by which they sense the level of exogenous FAs and possibly even distinguish among exogenous FAs that differ in chain length. We also do not know the mechanism of regulation of the pathway; in *S. cerevisiae* the level of ELO mRNA depends on the concentration of exogenous FAs (Toke and Martin, 1996).

Thus, the elongase pathway is ideally suited for parasites that migrate between vector and host and from one compartment to another within the vector or host. Depending upon the needs of a life-cycle stage and the availability of exogenous FAs, the ELO pathway can readily adapt to produce different products at the appropriate concentration. For example, we speculate that when BSFs invade the cerebrospinal fluid, where the ratio of total lipids to that in serum is ~0.003 (Lentner, 1981), the ELO pathway would be upregulated to produce not only myristate for GPI anchors, but also longer FAs for phospholipids.

Is the ELO Pathway Essential?

There are still unanswered questions concerning the ELO pathway. One critical question is whether the pathway is essential for growth of BSFs and therefore a candidate drug target. ELOs are clearly essential for PCF trypanosomes. RNAi silencing of EnCR, an enzyme in the ELO pathway, causes growth arrest, but normal growth is restored when the culture is supplemented with stearate. In the case of BSFs, *ELO* knockout lines grew normally in culture and were competent for infection in rats, suggesting that the pathway may not be required for growth in lipid-rich environments. However, it is possible that ELO specificities overlap sufficiently to compensate for knockout of a single ELO; another possibility is that the pathway is essential only in lipid-poor environments.

Substrates for Fatty Acid Synthesis

Although we know the origin of most of the substrates for the ELO pathway, the source of the butyryl-CoA primer is not yet known. The malonyl-CoA is formed by an acetyl-CoA carboxylase, probably in the cytosol, and NADPH can be produced either by oxidation of malate to pyruvate by a cytosolic malic enzyme (van Weelden et al., 2005) or by the pentose phosphate shunt, which may be localized in both the cytosol and the glycosomes (Duffieux et al., 2000). It is important to note that the ELO3 component of the pathway can act as a conventional ELO system, elongating exogenously supplied myristate to palmitate and stearate (Doering et al., 1993). In addition, ELO4 acts as a conventional polyunsaturated FA elongase in that it elongates arachidonoyl-CoA (C20:4) to C22:4.

It is striking that cell-free FA synthesis utilizing wild-type BSF membranes and a butyryl-CoA primer appears processive. Under these conditions, there are only low concentrations of intermediates detected, and the major product is C14 (Figure 5A, second panel). It is possible that when the entire pathway is operational, starting with butyryl-CoA, growing acyl-CoAs are channeled, with no release of intermediates. In contrast, use of C6- and C8-CoA primers (Figure 5A, third and fourth panels), results in nonprocessive synthesis of C14, with detection of all intermediates. The mechanistic explanation for these differences is not known, but a similar situation occurs in yeast (Dittrich et al., 1998). Substrate channeling might not only increase the efficiency of FA synthesis, but it also could impact GPI myristoylation. We found previously that the GPI remodeling enzymes in vitro can utilize FAs shorter than myristate; among shorter acyl chains, lauroyl-CoA and octanoyl-CoA are the preferred substrates (Morita and Englund, 2001). Myristate specificity in remodeling was previously assumed to be due to the lack of other available short-chain fatty acyl-CoAs. However, we now know that these other substrates (octanoyl-CoA to lauroyl-CoA) are present in the cell as intermediates in the FA synthesis pathway. Substrate channeling through the ELO pathway could prevent their incorporation into GPIs.

Conclusion

T. brucei and its trypanosomatid kin are unique among eukaryotes in that they synthesize FAs with a microsomal elongase pathway that is distinct from conventional type I and II synthases. This mechanism is a third type of FA synthesis. It is likely that the parasite's need for rapid adaptation to diverse host environments had a hand in the evolution of this piecemeal FA synthesis pathway.

EXPERIMENTAL PROCEDURES

Trypanosomes

Procyclic *T. brucei brucei* strain 29-13 (Wirtz et al., 1999) and derived cells were cultured at 27°C and 5% CO_2 in SDM-79 medium (Brun and Schonenberger, 1979). Bloodstream wild-type 427, 90-13, and knockout strains were cultured at 37°C and 5% CO_2 in HMI-9 medium (Hirumi and Hirumi, 1989). BSF lysates were made from trypanosomes grown in rats. Strain 90-13 was grown in nonirradiated retired male breeder rats while *Δelo1–4* strains were grown in lethally irradiated (1000 rad) rats. Trypanosomes were harvested by cardiac exsanguination when parasitemias reached 1–2 × 10^9 trypanosomes/ml. Blood

was transferred to an equal volume of Percoll (Sigma) containing 67 U/ml heparin, 8.6% sucrose, and 2.0% glucose (pH adjusted to 7.5 with solid HEPES) (Grab and Bwayo, 1982). After centrifugation (28,000 × g, 4°C, 15 min), the upper layer of trypanosomes was purified further by DE53 chromatography (Lanham and Godfrey, 1970). BSF and PCF crude cell membranes were prepared by washing cells in BBSG (50 mM bicine-Na [pH 7.5], 50 mM NaCl, 5 mM KCl, and 70 mM glucose). Cells (10^9/ml) were osmotically lysed on ice in 1 mM HEPES-KOH [pH 7.5], 1 mM EDTA, 1 μg/ml leupeptin, and 0.1 mM TLCK. After 5 min, an equal volume of 100 mM HEPES-KOH [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 300 mM sucrose, 1 μg/ml leupeptin, and 0.1 mM TLCK was added. Lysate aliquots were snap-frozen and stored at −80°C.

RNAi

A fragment from a region of the target open reading frame was cloned into pZJM, a vector for tetracycline-inducible RNAi (Wang et al., 2000). For ACP, the pZJM insert was 447 bp encoding the entire open reading frame. The ELO1 insert was 571 bp, starting with 5′ TTGCCAATAA; ELO3 insert was 415 bp, starting with 5′ ACACGGCCTT; and that for EnCR was 416 bp, starting with 5′ GGAGCTGGAG. pZJM transformants of *T. brucei* 29-13 were selected and cloned by limiting dilution. RNAi induction and Northern analyses were performed as described (Wang et al., 2000).

Intracellular Localization

The *ELO3* gene product was GFP-tagged at its C terminus. Its gene sequence was PCR amplified with 5′-MluI-HindIII-XbaI-ATGTTGATG AACTTCGGGGGCTCCT-3′ forward primer and 5′-BamHI-NheI-XhoI-TTTCTTTATTTCCGCTGCTACGTGTGGA-3′ reverse primer and inserted into the HindIII and NheI restriction sites of the pXSGFPM3FUS vector (Marchetti et al., 2000) for constitutive expression. The pXSGFPM3FUS NEO marker was also replaced with blasticidin drug marker using flanking AscI and PacI restriction sites. The vector was linearized with MluI for integration into the tubulin gene locus of wild-type 427 PCF trypanosomes. Transformants were selected with 10 μg/ml blasticidin. To stain ER, cells were incubated 30 min at 27°C and 5% CO_2 with 0.5 μM ER tracker Blue-White DPX (Molecular Probes) in BBSG.

Generating ELO Knockouts and Southern Analysis

ELO genes in BSF 427 strain were replaced by homologous recombination with pKO^{NEO} and pKO^{HYG} cassettes containing ~500 bp each of 5′ and 3′ sequences surrounding the open reading frame of the target gene (Lamb et al., 2001). Knockout transformants were cloned and drug selection was removed after gene deletions were confirmed by Southern analysis. For the latter, genomic DNA was isolated using Puregene DNA isolation kit (Gentra Systems), restriction digested, and fractionated. DNA was partially depurinated, transferred to GeneScreen Plus Nylon membrane (Perkin Elmer), and hybridized at 40°C to digoxigenin-labeled ELO gene sequences according to instructions in the DIG Probe Synthesis Kit and DIG Easy Hyb Kit (both from Roche).

Cell-Free Fatty Acid Synthesis

Thawed cell lysates were washed twice in HKML buffer (50 mM HEPES-KOH [pH 7.4], 25 mM KCl, 5 mM $MgCl_2$, 1 μg/ml leupeptin) and protein concentration was determined by the Bradford assay (Bio-Rad). Duplicate FA synthesis assays were conducted at 1 × 10^9 cell equivalents/ml in HKML, 1 mM DTT, 2 mM each of NADPH and NADH, 50 μM [2-^{14}C]malonyl-CoA (American Radiolabeled Chemicals, 55 mCi/mmol), and 50 μM acyl-CoA primer. Incubations were at 37°C for 30 min unless otherwise indicated. Lipids were extracted and fractionated on silica gel 60 HPTLC plates (EMD Chemicals Inc.) and visualized on Kodak BioMax MR film (Morita et al., 2000). Free FAs on TLC plates were quantitated using a Fujifilm BAS-2500 Phosphorimager. FA synthesis, measured as photostimulated luminescence, was corrected for local background and normalized for protein concentration. For the experiment in Figure 4B, lysates were washed once in HKML buffer, NADH was omitted, and 25 μM [2-^{14}C]-malonyl-CoA was used.

Fatty Acid Chain-Length Analysis

Chloroform/methanol/water (CMW) 10:10:3 extracts from cell-free FA synthesis reactions (~2.0 × 10^7 cell equivalents) or ^{3}H-labeled FA standards were mixed with carrier FAs (~0.2 nmol each of C8, C10, C12, C14, and C16). Samples were dried under N_2 gas, dissolved in 0.1 ml dry methanol containing 2% H_2SO_4 and 0.2% benzene, and incubated at 65°C for 2–16 hr to form methyl esters. Reactions were stopped with 100 μl water and FA methyl esters were extracted with 100 μl hexane (Fosbrooke and Tamir, 1968). Methyl esters of shorter fatty acids, especially C6, are lost during the hexane extraction. The hexane extracts were fractionated on C18 RP-TLC plates (Analtech) using CMW 5:15:1 v/v as mobile phase.

Argentation TLC

Silver nitrate (Sigma; 1.5 g) was dissolved in 12 ml of 50% acetonitrile and sprayed onto a 10 cm × 10 cm silica gel 60 plate using an atomizer. After drying in the hood, the plate was baked 30 min at 95°C. Methyl esters of C18:1, C18:2, C18:3, and C20:4 were used as standards and in a carrier mix (3.8% each of 16:1, 18:2, 18:3, and 20:4 methyl esters and 5% 18:1 methyl ester in hexane) that was spotted on the radiolabeled samples at the origin (1 μl/lane). The TLC was run 8 cm in dichloromethane. Standards were visualized by incubating the dried plate in a chamber equilibrated with iodine crystals. The plate was then sprayed with En^{3}Hance (NEN), dried, and visualized on film.

Measurement of Fatty Acid Synthesis in Cultured Parasites

Cells were grown either in normal medium (SDM-79 containing 10% FBS) or in low-lipid medium (SDM-79 supplemented with 10% delipidated FBS from Cocalico Biologicals). The latter medium contains ~20% of the lipids of normal medium. Stearate (35 μM) with α-cyclodextrin (350 μM) as carrier was delivered and RNAi was induced on day zero. Prior to metabolic labeling, cells were maintained in either normal medium or in low-lipid medium. After 5 days, cells (3–5 × 10^6/ml) were washed and resuspended at 1.2 × 10^8 cells/ml in delipidated FBS containing appropriate drugs. Cells (6 × 10^7) were then treated with 1.2 μCi L-[UL-^{14}C]threonine (Sigma, 155 mCi/mmol) for 4.3 hr (27°C, 5% CO_2). Cells were centrifuged, washed with PBS, and extracted in 0.8 ml CMW 10:10:3. Part of the CMW phase (1.5 × 10^7 cell equivalents) was used for chain-length analysis. Cells had normal morphology and motility after radiolabeling.

[^{14}C]Threonine Uptake

Procyclic *T. brucei* grown 5 days in normal or low-lipid medium were washed twice in BBSG and resuspended at 1.2 × 10^8 cells/ml in delipidated FBS containing appropriate drugs. Samples (350 μl) were treated with [^{14}C]threonine and incubated at 27°C under 5% CO_2. At times up to 4.5 hr, 50 or 100 μl aliquots were layered onto a mixture of 95 μl of dibutyl phthalate and 5 μl of paraffin oil, and cells were centrifuged through the oil mixture in a microfuge (Parsons and Nielsen, 1990). The upper aqueous phase was removed and the tube containing the remaining oil and cells was washed twice with 100 μl PBS. The oil mixture was then removed, and the cell pellet was washed with fresh oil mixture. Cells were resuspended in PBS, transferred to scintillation fluid, and counted.

Fatty Acid Synthesis in *T. cruzi* and *L. major*

T. cruzi CL Brener epimastigotes were grown in LIT medium supplemented with 10% FBS at 27°C (Zingales et al., 1997). *L. major* Friedlin promastigotes were grown in chemically defined M199+ medium (McCarthy-Burke et al., 1991) supplemented with 2 mM glutamine (Gibco) and 0.1 mM adenosine (Sigma) at 26°C. Membranes were prepared by

the method used for *T. brucei*, except *L. major* was lysed in double the volume of lysis buffer; thus lysates were half as concentrated when frozen. Cell-free FA synthesis assays were performed as before with NADPH as reducing agent (NADH was omitted). For FA chain-length analyses, carrier FAs were omitted.

ACKNOWLEDGMENTS

We thank Steve Beverly, Teresa Dunn, Dennis Grab, Dan Lane, Yasu Morita, Shilpi Paul, Terry Shapiro, and Paul Watkins for discussions. We thank Barbara Burleigh and Anne Chessler for *T. cruzi* lysates and Dennis Dwyer for *L. major* cells and media. We thank Gokben Yildirir for technical assistance and members of our laboratory for support. We thank Wade Gibson for use of the phosphorimager. This work was supported by a grant from the National Institutes of Health (AI21334).

Received: April 17, 2006
Revised: May 15, 2006
Accepted: June 12, 2006
Published: August 24, 2006

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